J. Biochem. **141**, 181–192 (2007)
doi:10.1093/jb/mvm018

Orphan Nuclear Receptor Nur77 Accelerates the Initial Phase of Adipocyte Differentiation in 3T3-L1 Cells by Promoting Mitotic Clonal Expansion

Toshio Fumoto, Tomohiro Yamaguchi, Fumiko Hirose and Takashi Osumi*

Graduate School of Life Science, Himeji Institute of Technology, University of Hyogo, Kamigori, Hyogo 678-1297, Japan

Received November 21, 2006; accepted November 27, 2006; published online December 13, 2006

Nur77 is an orphan member of the nuclear receptor superfamily that is expressed in various types of cells and mediates diverse biological processes. Although Nur77 mRNA is induced in the early stage of adipogenesis of 3T3-L1 cells, its roles are not known. To address this issue, we closely inspected the expression of Nur77 mRNA and protein during differentiation of 3T3-L1 cells. Nur77 was induced rapidly and transiently at both mRNA and protein levels only in the initial phase of differentiation induction, and localized almost exclusively in the nuclei. Isobutylmethylxanthine was essential for the induction of Nur77 protein, acting by at least in part protecting the protein from rapid degradation by proteasome. Nur77 siRNA resulted in delayed adipogenesis in 3T3-L1, accompanied by retarded mitotic clonal expansion. These effects were mediated at least partly by decreased expression of cyclins D and E. Constitutive expression of Nur77 inhibited adipogenesis of 3T3-L1, accompanied by enhanced expression of cyclin D1 and prolonged mitotic clonal expansion. Moreover, constitutive expression of Nur77 inhibited, but transient induction of Nur77 promoted, adipogenesis in NIH-3T3 cells. These results suggest that Nur77 accelerates adipocyte differentiation by regulating cell cycle progression and the rapid and transient induction is crucial for its action.

Key words: adipocyte differentiation, cyclin, clonal expansion, NIH-3T3, Nur77, 3T3-L1.

Abbreviations: PPAR, peroxisome proliferator-activated receptor; C/EBP, CCAAT/enhancer binding protein; Dex, dexamethasone; IBMX, isobutylmethylxanthine; PBS, phosphate-buffered saline; RT, reverse transcription; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; BrdU, bromodeoxyuridine; LLL, carbobenzoxy-leucyl-leucyl-leucinal; LLN, *N*-acetyl-leucyl-leucyl-norleucinal; KLF, Krüppel-like factor.

INTRODUCTION

Adipose tissue plays essential roles in energy homeostasis as not only the primary depot for energy stores but also as a secretory organ of various signalling molecules. Understanding the mechanism of adipose tissue development is becoming important because of the increasing incidence of obesity and multiple metabolic disorders associated with it (*1*).

Studies of the molecular mechanisms that regulate adipocyte differentiation have been greatly advanced by the use of preadipocyte cell lines such as 3T3-L1. Proliferating preadipocytes become growth-arrested at the confluence by contact inhibition. Upon treatment with a hormonal cocktail composed of dexamethasone (Dex), isobutylmethylxanthine (IBMX) and insulin, the growth-arrested cells re-enter the cell cycle. After a few rounds of cell division, known as mitotic clonal expansion, these cells are arrested for proliferation again, accumulate lipid droplets, and acquire biochemical and morphological characteristics of mature adipocytes. These processes require highly organized and precisely controlled expression of a cascade of transcription factors. The rapid and transient induction of the CCAAT/enhancer binding proteins (C/EBP) β and α is one of the earliest steps in this process. These transcription factors bind to specific sequences in the promoters of genes encoding PPARγ and C/EBPα, hence inducing their expression. Once expressed, PPARγ and C/EBPα enhance each other's production and act synergistically in the transcriptional activation of a variety of adipocyte-specific genes (*2*). Although the central involvement of these factors in adipogenesis has been demonstrated with both cultured cells and knockout animals (*3–5*), involvement of other regulatory molecules in this highly orchestrated transcriptional program is also coming apparent (*6, 7*).

Nur77 (also known as NGFI-B, TR3 and N-10) is an orphan member of the nuclear receptor superfamily. Nur77 is expressed in many types of cells in response to diverse stimuli, and involved in multiple cellular events such as proliferation, differentiation and apoptosis (*8*). Previous studies showed that expression of Nur77 is induced in the early stage of adipogenesis of 3T3-L1 cells (*9–11*). However, the roles of Nur77 in this process are yet to be defined.

*To whom correspondence should be addressed. Tel: +81-791-58-0192, Fax: +81-791-58-0193, E-mail: osumi@sci.u-hyogo.ac.jp

© 2006 The Japanese Biochemical Society.

Downloaded from http://jb.oxfordjournals.org/ at Universidade Federal do Pará on September 29, 2012

Table1. **Sequences of PCR primers.**

	Forward primer	Reverse primer
Nur77	5′-CCACCTCTCCGAACCGTGACA-3′	5′-GAGAAGATTGGTAGGGGAGGC-3′
PPARγ2	5′-GAGGAATTCATGGGTGAAACTCTGGGAGATTC-3′	5′-GACGGATCCCTCAATGGCCATGAGGGAGTTAG-3′
C/EBPα	5′-ACAACATCGCGGTGCGCAAGA-3′	5′-TGCCATGGCCTTGACCAAGGAG-3′
C/EBPβ	5′-GGCCAAGAAGACGGTGGACAA-3′	5′-TTCTTCTGCAGCCGCTCGTTC-3′
C/EBPδ	5′-CCACGACTCCTGCCATGTACGA-3′	5′-GGCCGCTTTGTGGTTGCTGTT-3′
Adipsin	5′-ACGGATGACGACTCTGTGCAG-3′	5′-GGATGACACTCGGGTATAGAC-3′
aP2	5′-GAAGACAGCTCCTCCTCGAAG-3′	5′-TAAACTCTTGTGGAAGTCACG-3′
Cyclin D1	5′-GACACCAATCTCCTCAACGAC-3′	5′-TTCATCTTAGAGGCCACGAAC-3′
Cyclin D2	5′-CAGAACCTGTTGACCATCGAG-3′	5′-CCACTTCAGCTTACCCAACAC-3′
Cyclin D3	5′-CCTTGATTCTGCACCGCCTGTCTCT-3′	5′-TCTCCCTGAGGGCAGCTTCGATCTG-3′
Cyclin E1	5′-TTCTTCTGGATTGGCTGATGG-3′	5′-GTTGACATAGGCCACTTGGAC-3′
Cyclin E2	5′-AGAAAAGAGGAGATCACCAAG-3′	5′-GCCAGTCTAAAAGTATTGACC-3′
36B4	5′-TTCGTGTTCACCAAGGAGGAC-3′	5′-ATGATCAGCCCGAAGGAGAAG-3′
RpS12	5′-GGAGGTGTAATGGACGTCAAC-3′	5′-TTACAGAGGCCTACCCATTCC-3′

Here we demonstrate that expression of Nur77 protein is tightly restricted in the very early stage of adipogenesis of 3T3-L1 cells. Modulation of Nur77 expression by knockdown or overexpression resulted in impaired adipogenesis in 3T3-L1 cells, accompanied by modified mitotic clonal expansion. Moreover, transient induction of Nur77 promoted adipogenesis in NIH-3T3 cells. These results indicate that Nur77 is involved in adipocyte differentiation through regulating cell cycle progression.

EXPERIMENTAL PROCEDURES

Cell Culture—3T3-L1 cells were passaged in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). Preadipocytes were induced for differentiation 2 days after confluence by exposure to DMEM containing 10% FBS, 1 μM Dex, 0.5 mM IBMX, and 5 μg/ml insulin for 2 days. The cells were further cultured in DMEM containing 10% FBS and 5 μg/ml insulin, with changing the medium every 3 days. NIH-3T3 cells were maintained in DMEM containing 10% FBS. For differentiation experiments, medium was replaced with DMEM containing 10% FBS, 1 μM Dex, 0.5 mM IBMX, 10 μg/ml insulin, and 0.5 μM BRL49653, for 2 days after confluence. After another 2 days, the medium was replaced by DMEM containing 10% FBS, 10 μg/ml insulin, and 0.5 μM BRL49653, and was changed every 3 days. 293FT cells (Invitrogen) were maintained according to the manufacturer's instructions.

For Oil Red-O staining, cells were fixed for 20 min with 3.3% formalin and for 1 h with 10% formalin in PBS. After being washed three times with water, fixed cells were stained with 0.3% Oil-Red O for 1 h at room temperature. Cells were then washed three times with water, dried and photographed.

RNA Isolation and Reverse Transcription (RT)-PCR—Total RNA was prepared using a QIAzol Lysis Reagent and a RNeasy minikit, according to the manufacturer's instructions (QIAGEN). RT was performed with M-MLV reverse transcriptase (Invitrogen) using 1 μg of total RNA and a mixture of downstream primers (2 pmol each), as recommended by the manufacturer. PCR was performed with rTaq DNA polymerase (Takara) in a volume of 25 μl containing 10 pmol each of the upstream and downstream primers, and 1 μl of RT product, following the manufacturer's instructions. The PCR conditions for ribosomal protein 36B4 were 94°C for 2 min, followed by 23 cycles of 30 s at 94°C, 30 s at 55°C and 30 s at 72°C. The number of PCR cycles and the annealing temperature were adjusted for each gene. The reaction products were separated in 2 or 3% agarose gels, the bands were detected with a fluorescence imaging analyzer FLA3000, and their intensities were analysed using the software, Image Gauge (Fuji). 36B4 was used as a loading control in time-course experiments spanning more than 2 days, whereas RpS12, another ribosomal protein gene, was used for experiments within 48 h. This was because 36B4 expression changed in a very early stage of differentiation induction. Reproducibility was confirmed for most experiments by two or more assays. Specific oligonucleotide primers used are listed in Table 1.

Real-time RT-PCR—For quantitative assessment of mRNA expression levels, real-time RT-PCR was performed. For glyceraldehyde-3-phosphate dehydrogenase (GAPDH), Rodent GAPDH Control Reagents (Applied Biosystems) were used. Primers and TaqMan probes for other genes were designed using the Primer Express 2.0 (Applied Biosystems). Real-time PCR analysis was performed in an ABI Prism 7700 sequence detection system (Applied Biosystems), using TaqMan One-Step RT-PCR Master Mix Reagents (Applied Biosystems). Reactions were carried out on MicroAmp Optical 96-well plates (Applied Biosystems) using 12.5 ng of total RNA, 900 nM of each forward and reverse primers, and 250 nM of TaqMan probe in a total volume of 25 μl. For GAPDH, 100 nM of each forward and reverse primers, and 200 nM of TaqMan probe were used. The reaction comprised an initial reverse transcription step with incubation at 48°C for 30 min, followed by AmpliTaq Gold enzyme activation at 95°C for 10 min, and finally PCR amplification performed at 95°C for 15 s and 60°C for 1 min for 40 cycles. The probe and primer sets used are summarized in Table 2. The relative expression was normalized with that of 36B4 or GAPDH. Means for two independent samples are shown together with standard deviations.

Downloaded from http://jb.oxfordjournals.org/ at Universidade Federal do Pará on September 29, 2012

Table 2. **Sequences of primers and Taqman probes for real-time PCR.**

	Forward	5′-TGACCCAATGGTTGCTGATTAC-3′
PPARγ	Reverse	5′-GAAGGTTCTTCATGAGGCCTGTT-3′
	Probe	5′-[FAM]-CCTGAAGCTCCAAGAATACCAAAGTGCG-[TAMRA]-3′
	Forward	5′-TGGACAAGAACAGCAACGAGTAC-3′
C/EBPα	Reverse	5′-CGGTCATTGTCACTGGTCAACT-3′
	Probe	5′-[FAM]-CAAACAACGCAACGTGGAGACGCA-[TAMRA]-3′
	Forward	5′-AAGCTGAGCGACGAGTACAAGA-3′
C/EBPβ	Reverse	5′-GTCAGCTCCAGCACCTTGTG-3′
	Probe	5′-[FAM]-CGCGAGCGCAACAACATCGC-[TAMRA]-3′
	Forward	5′-CCAGTTCACAGCTGCCAATGAGTT-3′
Perilipin	Reverse	5′-GGTATTGAAGAGCCGGGATCTT-3′
	Probe	5′-[FAM]-CCTGCAGAGGCCTGGACCACCTG-[TAMRA]-3′
	Forward	5′-GCGCCCTCCGTATCTTACTTC-3′
Cyclin D1	Reverse	5′-CTCACAGACCTCCAGCATCCA-3′
	Probe	5′-[FAM]-TGCCATCCATGCGGAAAATCG-[TAMRA]-3′
	Forward	5′-CTGCTGCCGCCTTATGTCAT-3′
Cyclin E2	Reverse	5′-CAAAAGGCACCATCCAGTCTACA-3′
	Probe	5′-[FAM]-TTTGGAATGGGATGACATCTCGGAA-[TAMRA]-3′
	Forward	5′-GAGGCACCATTGAAATTCTGAGT-3′
36B4	Reverse	5′-TGTCAAACACCTGCTGGATGA-3′
	Probe	5′-[FAM]-ATAAAGACTGGAGACAAGGTGGGAGCCA-[TAMRA]-3′

Western Blotting and Immunofluorescence Microscopy—Cultured cells were washed with phosphate-buffered saline (PBS) twice and were directly dissolved in heated SDS-PAGE sample buffer. After being passaged 10 times through 29G needles, the cell lysates were subjected to SDS-PAGE using 10% gels and transferred to nitrocellulose membranes (Millipore). Membranes were blocked for 1 h in PBS containing 0.5% Tween 20 and 5% skimmed milk at 4°C, and incubated for 1 h at room temperature with primary antibodies appropriately diluted with PBS containing 0.5% Tween 20 and 1% skimmed milk. After being washed with PBS containing 0.5% Tween 20 three times, the membranes were incubated with peroxidase-conjugated secondary antibody diluted in PBS containing 0.5% Tween 20 and 2% skimmed milk for 1 h at room temperature. After washing as described earlier, bands were visualized with ECL Western blotting detection reagents (Amersham). In the assay to confirm expression of Nur77 driven by human metallothionein IIA promoter, blocking and incubation with the antibody were carried out in PBS containing 0.5% Tween 20 and 1% skimmed milk, and bands were visualized with SuperSignal West Femto Kit (PIERCE).

For immunofluorescence microscopy, cells were grown on coverslips, and induced for differentiation. Cells were washed with PBS three times and fixed with 4% paraformaldehyde in PBS for 20 min at room temperature. After washing with PBS three times, fixed cells were permeabilized with 0.2% Triton X-100 in PBS for 20 min at room temperature. After being washed three times with PBS, cells were blocked with 2% bovine serum albumin in PBS for 20 min at room temperature, and then overnight at 4°C. Cells were incubated with a primary antibody for 1 h at 37°C, washed three times with PBS, and incubated with an Alexa Fluor-conjugated secondary antibody for 1 h at 37°C. After being washed three times with PBS, nuclei were stained with Hoechst 33258 (Sigma) for 10 min at room temperature. After being washed three times with PBS, cells were mounted with 90% glycerol in PBS containing 25 mg/ml 1,4-diazabicyclo-[2.2.2]octane and observed under a fluorescence microscope (KEYENCE, or as described previously (*12*)). For staining mitochondria, cells grown on coverslips were induced for differentiation with standard differentiation medium containing 25 nM MitoTracker Orange (Molecular Probes). For bromodeoxyuridine (BrdU) incorporation, cells grown on coverslips were induced for differentiation with the standard protocol for various periods. Cells were then incubated in the standard differentiation medium supplemented with 10 μM BrdU for 30 min at 37°C, washed with PBS three times, and fixed with ethanol fixative (50 mM glycine-HCl (pH 2.0) in 70% ethanol) for 1 h at −20°C, and blocked with 2% bovine serum albumin in PBS.

The following antibodies were used: mouse anti-Nur77 monoclonal antibody (BD Biosciences); rabbit anti-LDH antibody (kindly provided by Dr N. Usuda); goat anti-LDH polyclonal antibody (Abcam); rabbit anti-C/EBPβ polyclonal antibody (Santa Cruz); mouse anti-BrdU monoclonal antibody (Roche); horseradish peroxidase-linked sheep anti-mouse IgG antibody (Amersham Biosciences); horseradish peroxidase-linked donkey anti-rabbit IgG antibody (Amersham Biosciences); horseradish peroxidase-linked donkey anti-goat IgG antibody (Santa Cruz); Alexa Fluor 594-conjugated anti-mouse IgG antibody (Molecular Probes); Alexa Fluor 488-conjugated anti-mouse IgG antibody (Molecular Probes); and Alexa Fluor 488-conjugated anti-rabbit IgG antibody (Molecular Probes).

Plasmid Construction—Nur77 cDNA was obtained by RT-PCR using total RNA prepared from 3T3-L1 cells 1 h after induction of differentiation. The 5′ region of Nur77 cDNA was amplified using KOD

Downloaded from http://jb.oxfordjournals.org/ at Universidade Federal do Pará on September 29, 2012

plus (TOYOBO) with a forward primer, CGGGGTACCA TGCCCTGTATTCAAGCTCAA, and reverse primer, TTG GGTTTTGAAGGTAGCCGG. The 3′ region of Nur77 cDNA was similarly amplified with a forward primer, CCACCTCTCCGAACCGTGACA, and reverse primer, TCAGAAAGACAATGTGTCCAT. The amplified fragments were subcloned into the EcoRV site of pBluescript II KS (–) (STRATAGENE). The 5′ fragment of Nur77 cleaved with NotI and HaeII and the 3′ fragment of Nur77 cleaved with BamHI and HaeII were inserted between the NotI and BamHI sites of CSII-EF-MCS-IRES2-Venus (kindly provided by Dr H. Miyoshi) to generate a lentiviral expression vector CSII-EF-Nur77-IRES2-Venus. For constructing an inducible lentiviral expression vector, CSII-EF-MCS-IRES2-Venus was partially digested with EcoRI and XhoI, and self-ligated to generate a vector CSII-EF-MCS-IRES2-Venus-EX, in which EcoRI and XhoI sites are unique. Human metallothionein IIA promoter (a gift from Dr S. Taketani) from which the glucocorticoid response elements were removed was inserted between the AgeI and XhoI sites of CSII-EF-MCS-IRES2-Venus-EX to generate a lentiviral expression vector CSII-MT-MCS-IRES2-Venus. This vector contains the human metallothionein IIA promoter instead of the human elongation factor (EF)-1α subunit gene promoter. Nur77 cDNA was inserted into the BamHI site of CSII-MT-MCS-IRES2-Venus to generate a lentiviral expression vector CSII-MT-Nur77-IRES2-Venus.

For small interfering RNA (siRNA) experiments, oligonucleotides with the following sequences of top and bottom strands were chemically synthesized. Nur77 RNAi1: top, CACCGTGACACTTCCGGCATTCT CCACACCAGAATGCCGGAAGTGTCAC, and bottom, AAAAGTGACACTTCCGGCATTCTGGTGTGGAGAATGC CGGAAGTGTCAC. Nur77 RNAi2: top, CACCGCGCCA AGTACATCTGCCTCCACACCAGGCAGATGTACTTGGC GC, and bottom, AAAAGCGCCAAGTACATCTGCCTG GTGTGGAGGCAGATGTACTTGGCGC. Scramble: top, CACCGCGCGCTTTGTAGGATTCGCCACACCCGAATCC TACAAAGCGCGC, and bottom, AAAAGCGCGCTTTGTA GGATTCGGGTGTGGCGAATCCTACAAAGCGCGC. To create entry clones, the top and bottom strands of oligonucleotide were annealed and ligated into pENTR/U6 (Invitrogen). To generate lentiviral vectors expressing siRNAs, LR recombination reaction was performed between the entry clones and CS-RfA-EG (kindly provided by Dr H. Miyoshi), using Gateway LR Clonase (Invitrogen) according to the manufacturer's instruction.

Preparation of Recombinant Lentiviruses and Lentiviral Infection—To generate viral stocks, 293FT cells were cotransfected with one of the lentivirus expression plasmids, pCMV-VSV-G-RSV-Rev and pCAG-HIVgp (kindly provided by Dr H. Miyoshi), using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. After 12 h of transfection, the medium was changed, and the cells were incubated for 24 h. The supernatants containing the recombinant viruses were collected, filtered through 0.45 μm filters, and stored at −80°C. The infection of 3T3-L1 and NIH-3T3 cells was carried out by incubating 30% confluent cells with growth medium supplemented with a 50% lentivirus stock and 6 μg/ml polybrene for 8 h. We confirmed that more than 90% of cells were infected as judged by the expression of Venus (a marker embedded in the vector), monitored by fluorescence microscopy.

RESULTS

Nur77 is Induced in the Very Early Stage of Adipocyte Differentiation of 3T3-L1 Cells—Previous works showed that Nur77 mRNA is induced in the early stage of differentiation of 3T3-L1 cells (*9–11*). To investigate the role of Nur77 during adipocyte differentiation, we first examined the expression of Nur77 at both mRNA and protein levels. 3T3-L1 cells were induced to differentiate into adipocytes by treating the cells with a standard hormonal cocktail. As shown in Fig. 1A, Nur77 mRNA was induced rapidly, peaking 1 h after induction, declined quickly to the basal level at 48 h, and kept thereafter at a low constant level. More strikingly, Nur77 protein was induced rapidly and diminished quickly, coming undetectable within 9 h (Fig. 1B). Because subcellular localization of Nur77, nuclear or mitochondrial, has been suggested to be a crucial determinant for its function (*13*), we next examined the intracellular distribution of Nur77 during adipocyte differentiation by immunofluorescence microscopy. As shown in Fig. 1C, Nur77 was localized almost exclusively in the nuclei. We also noticed that Nur77 exhibited punctate distribution within the nuclei. At higher magnification, this pattern was distinct from that of the bright dots revealed by Hoechst staining (Fig. 1D). The latter coincided with the punctate distribution of C/EBPβ, an adipogenic transcription factor induced in early adipogenesis and enriched in centromeres after re-entry of the 3T3-L1 cells into S phase (*14*). These results suggest that Nur77 plays a role in the early stages of adipogenesis as a transcription factor.

Because Nur77 was originally cloned as an immediate early gene that is induced by serum in mouse fibroblasts (*15, 16*), we analysed the regulatory mechanism of Nur77 expression in 3T3-L1 cells. To clarify the respective roles of differentiation inducers on the expression of Nur77, we exposed 3T3-Ll cells to hormonal cocktails in which different components were systematically omitted. As shown in Fig. 1E, changing of the medium, which supplied fresh FBS, was by itself sufficient for the induction of Nur77 mRNA (compare lanes 1 and 9). Dex slightly repressed (lanes 2 and 3, and 8 and 9), whereas insulin slightly enhanced the expression of Nur77 mRNA (lanes 2 and 5, and 6 and 9). In contrast, IBMX enhanced the expression of Nur77 mRNA approximately three fold (lanes 2 and 4, and 7 and 9). On the other hand, expression of Nur77 protein was completely dependent on IBMX (Fig. 1F, lanes 2, 3, 5 and 7). These results suggest that Nur77 expression is regulated both transcriptionally and post-transcriptionally in 3T3-L1 cells, IBMX being likely to be involved in both steps. Accordingly, we next examined the effect of IBMX on Nur77 protein degradation. One hour after induction of differentiation, culture medium was replaced with the

Downloaded from http://jb.oxfordjournals.org/ at Universidade Federal do Pará on September 29, 2012

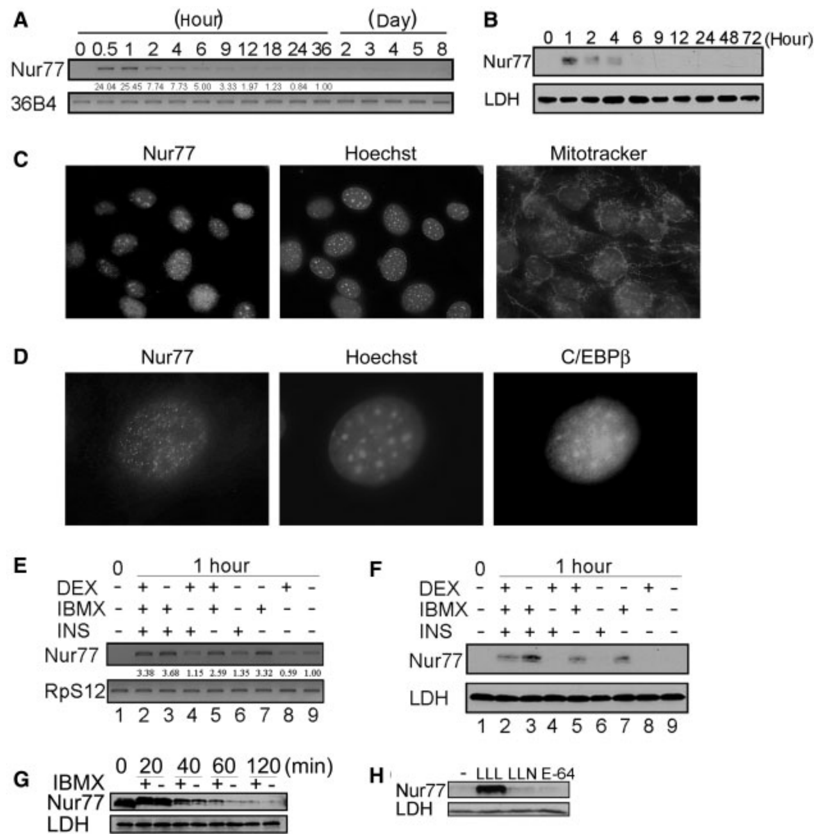

Fig. 1. **Expression of Nur77 is restricted in the early phase of 3T3-L1 adipocyte differentiation.** (A) Time course of Nur77 mRNA expression during 3T3-L1 adipocyte differentiation. 3T3-L1 cells were induced to differentiate by the standard protocol. Total RNA extracted at times indicated was subjected to RT-PCR. 36B4 was used as a loading control. Values below the picture of Nur77 indicate the relative band intensities estimated by densitometric scanning, which were normalized with 36B4. (B) Time course of Nur77 protein expression during 3T3-L1 adipocyte differentiation. Whole cell extracts prepared at times indicated were immunoblotted with an anti-Nur77 polyclonal antibody. LDH was used as a loading control. (C) Subcellular localization of Nur77 during 3T3-L1 adipocyte differentiation. 3T3-L1 cells cultured on coverslips were induced to differentiate using standard differentiation medium containing MitoTracker Orange for 1 h. Cells were fixed, immunostained with anti-Nur77 antibody, and counterstained for the nuclei with Hoechst 33342. (D) Intranuclear distribution of Nur77 during 3T3-L1 adipocyte differentiation. 3T3-L1 cells cultured on coverslips were induced to differentiate by the standard protocol for 1 h. Cells were fixed, double immunostained with anti-Nur77 and anti-C/EBPβ antibodies, and counterstained with Hoechst 33342. (E) Effects of the components of differentiation cocktail on Nur77 mRNA expression. 3T3-L1 cells were treated with various combinations of differentiation inducers for 1 h. Nur77 mRNA was measured by RT-PCR. Ribosomal protein S12 was used as a loading control. Values below the picture of Nur77 indicate the relative band intensities estimated by densitometric scanning, which were normalized with RpS12. (F) Effects of the components of differentiation cocktail on Nur77 protein expression. Cells were treated as in E, and the whole cell extracts were analysed by Western blotting. (G) Effect of IBMX on Nur77 protein degradation. One hour after induction of differentiation, culture medium was replaced with the standard differentiation medium with or without IBMX. Cycloheximide (10 μM) was added simultaneously. Cells were incubated for periods indicated, and the whole cell extracts were subjected to Western blotting. (H) Involvement of proteasome in the degradation of Nur77 protein. One hour after induction of differentiation, 3T3-L1 cells were treated with 25 μM LLL, 25 μM LLN, or 50 μM E-64, in the presence of 10 μM cycloheximide. Cells were incubated for 2 h, and whole cell extracts were subjected to Western blotting.

standard differentiation medium with or without IBMX. Cycloheximide was added simultaneously. Cells were incubated for the periods indicated, and whole cell extracts were analysed for Nur77 protein by Western blotting. As shown in Fig. 1G, in the presence of IBMX Nur77 protein was degraded more slowly than in the absence of IBMX, indicating that IBMX protects Nur77 protein from degradation at least partially. To assess the mechanism of degradation of Nur77 protein further, we next examined the effect of a proteasome inhibitor on the Nur77 protein level. One hour after induction of differentiation, 3T3-L1 cells were treated in the presence of cycloheximide with carbobenzoxy-leucyl-leucyl-leucynal (LLL), an potent inhibitor of proteasome and calpain, *N*-acetyl-leucyl-leucyl-norleucinal (LLN) that inhibits calpain efficiently, whereas proteasome only slightly, and E-64, a highly selective cysteine protease inhibitor. Induction of differentiation was continued for further 2 h and whole cell extracts were analysed by Western blotting. As shown in Fig. 1H, only LLL inhibited the degradation of Nur77 significantly. Thus, Nur77 is rapidly degraded by proteasome on the one hand, and on the other its synthesis is enhanced upon the differentiation induction. These results indicate that

Downloaded from http://jb.oxfordjournals.org/ at Universidade Federal do Pará on September 29, 2012

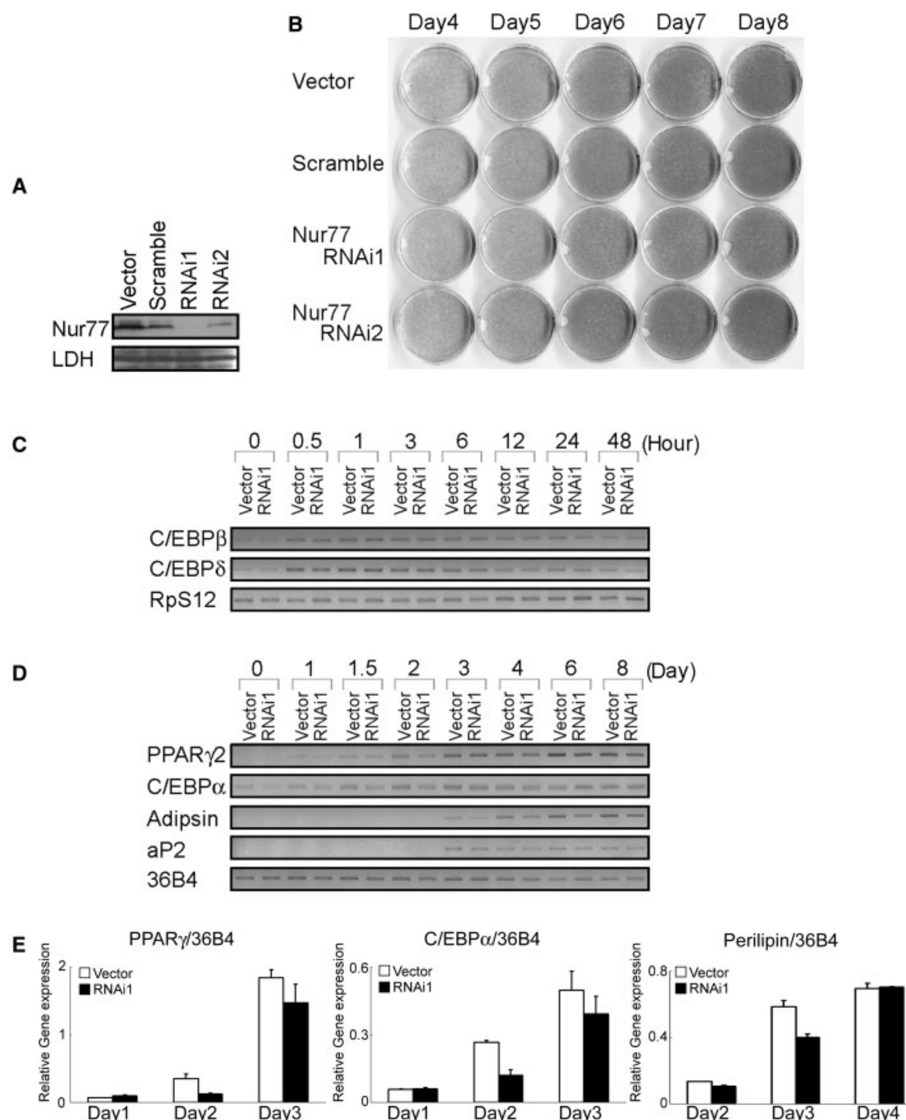

Fig. 2. **Effect of Nur77 gene silencing on adipocyte differentiation of 3T3-L1 cells.** (A) siRNA-mediated gene silencing of Nur77. 3T3-L1 cells were infected with lentiviruses directing the expression of siRNAs for Nur77, a scramble control virus, or an empty virus. Infected cells were expanded, and induced for differentiation. Whole cell extracts prepared 3 h after induction of differentiation were analysed by immunoblotting with an anti-Nur77 antibody. LDH was used as a loading control. (B) Effect of Nur77 gene silencing on the development of lipid droplets. 3T3-L1 cells infected as in A were induced for differentiation, and cultured until day 8. On days 4–8, cells were stained with Oil Red-O. (C, D, E) Effect of Nur77 gene silencing on the expression of adipogenic marker genes. Nur77 RNAi1 and vector control cells were induced for differentiation. Gene expression of the adipogenic markers was estimated by RT-PCR (C, D) and real-time RT-PCR (E). Ribosomal protein S12 or 36B4 was used as an internal control.

Nur77 is tightly regulated during 3T3-L1 adipocyte differentiation, and its protein expression is limited in the very early stage.

Nur77 Gene Silencing with siRNA Results in Delayed Adipogenesis in 3T3-L1 Cells—To determine whether Nur77 is required for adipocyte differentiation of 3T3-L1 cells, we next knocked down the expression of Nur77 using lentiviruses specifying siRNAs against Nur77 mRNA. Two oligonucleotide pairs encoding small hairpin RNAs designed to match different regions of Nur77 mRNA were cloned into the CS-RfA-EG lentiviral plasmid. The recombinant plasmids were transfected together with the packaging plasmids into 293FT cells to produce lentiviruses that direct the siRNA. 3T3-L1 cells were infected with the recombinant viruses, expanded, and induced to differentiate. To confirm efficiency of knockdown, whole cell extracts prepared at 3 h after induction of differentiation were analysed by immunoblotting. In Nur77 RNAi1 cells, expression of Nur77 was almost completely blocked, whereas Nur77 RNAi2 cells exhibited about half of the control expression level (Fig. 2A). Oil Red-O staining of the cells on days 4–8 of differentiation demonstrated that Nur77 RNAi1 cells accumulated slightly but significantly less lipid

Downloaded from http://jb.oxfordjournals.org/ at Universidade Federal do Pará on September 29, 2012

than control cells at all time points, reproducibly (Fig. 2B). As compared with control cells, lipid accumulation in Nur77 RNAi1 cells was delayed by approximately a day. Nur77 RNAi2 cells accumulated almost the same level of lipid as control cells, indicating that the residual expression of Nur77 is sufficient.

Next, we determined the expression levels of adipogenic marker genes in Nur77 RNAi1 cells by RT-PCR. The expression levels of C/EBPβ and C/EBPδ, which were induced in the kinetics similar to that of Nur77, were not affected by Nur77 knockdown at all time points examined (Fig. 2C). On the other hand, the expression of PPARγ2 which was induced later than Nur77, was significantly impaired on day 2 in Nur77 RNAi1 cells, and also considerably at later time points. Similarly, the induction of C/EBPα was significantly impaired in Nur77 RNAi1 cells on days 2 and 3, though coming comparable to that of control cells on day 6. On the other hand, the induction of adipsin and aP2 were retarded in the Nur77 RNAi1 cells (Fig. 2D). To confirm these results, we performed real-time RT-PCR. Expression level of C/EBPβ was not affected by Nur77 knockdown (data not shown). On the other hand, expression of PPARγ and C/EBPα was significantly impaired in Nur77 RNAi1 cells in the early points of their induction. In addition, induction of perilipin, a lipid droplet associated protein whose expression is induced by PPARγ during adipocyte differentiation, was retarded in Nur77 RNAi1 cells (Fig. 2E). These results indicate that Nur77 is involved in differentiation of 3T3-L1 cells, acting upstream of PPARγ2 and C/EBPα.

Nur77 Gene Silencing with siRNA Results in Delayed Mitotic Clonal Expansion—Expression of Nur77 is limited in the very early stage of differentiation in 3T3-L1, when the mitotic clonal expansion is triggered. In the course of experiments, we noted that clonal expansion itself was likely to be delayed in Nur77 RNAi1 cells as judged by morphological observation. That is, normal 3T3-L1 cells become slender and form reticular networks in this process, whereas Nur77 RNAi1 cells were retarded in exhibiting such morphological changes during the experimental period. Accordingly, we evaluated the effects of Nur77 gene silencing on mitotic clonal expansion. Upon induction of differentiation, growth-arrested 3T3-L1 cells synchronously re-enter the cell cycle and undergo about two rounds of cell divisions. Along with this, the cell numbers are increased about 2-fold within 48 h, and about 4-fold within 72 h, after differentiation induction (*17*). Nur77 RNAi1 and control cells were induced to differentiate and the cell numbers were counted every 6 h. Increase in the cell number of Nur77 RNAi1 cells was slightly but significantly delayed as compared with that of control cells, though Nur77 gene silencing did not completely block mitotic clonal expansion, hence the cell number being approximately doubled in the first 48 h (Fig. 3A). To assess the cell cycle progression more precisely, we next monitored DNA synthesis by BrdU labelling. It was reported that upon induction of differentiation, growth-arrested 3T3-L1 cells synchronously re-enter the cell cycle and cross the G1-S checkpoint between 12 and 16 h after induction (*18*).

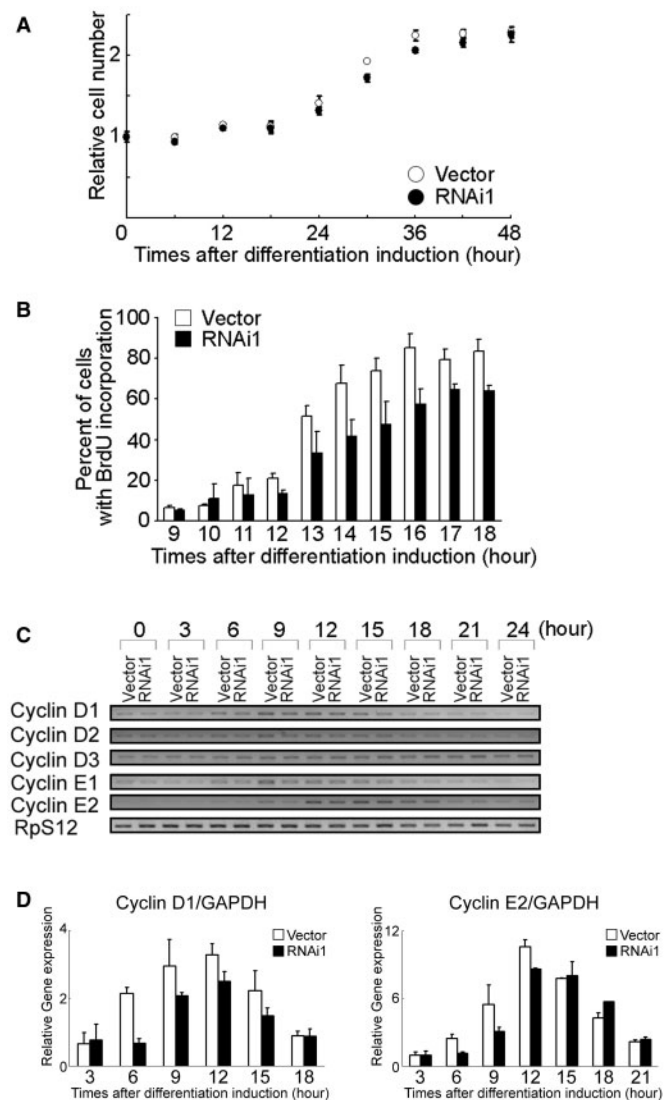

Fig. 3. **Effect of Nur77 gene silencing on mitotic clonal expansion.** (A) Effect of Nur77 gene silencing on the cell growth in the early phase of mitotic clonal expansion. 3T3-L1 cells infected with the lentivirus specifying siRNA1 for Nur77 or an empty vector were induced to differentiate. Cells were trypsinized from culture dishes and collected by centrifugation, and cell numbers were counted at different times after induction of differentiation. At the time of induction, there was no difference in cell numbers between Nur77 RNAi1 and vector control cells, which were taken as 1. Relative cell numbers at times indicated are shown as the means of three culture dishes together with standard deviations. (B) Effect of Nur77 gene silencing on the re-entry into S phase during mitotic clonal expansion. Knockdown and control cells were pulsed with BrdU for 30 min at different times after induction. BrdU incorporated and nuclei were visualized with an anti-BrdU antibody and Hoechst 33342, respectively. Percentages of BrdU-positive cells were determined by immunofluorescence microscopy. Results are given as the means of at least 200 cells in three independent experiments, together with standard deviations. (C, D) Effect of Nur77 gene silencing on the expression of cyclins, in the initial phase of mitotic clonal expansion. Total RNA was prepared from cells at times indicated after induction, and subjected to RT-PCR (C) and real-time RT-PCR (D). Ribosomal protein S12 or GAPDH was used as an internal control.

Downloaded from http://jb.oxfordjournals.org/ at Universidade Federal do Pará on September 29, 2012

Nur77 RNAi1 cells and control cells were pulse-labelled for 30 min with BrdU every 1 h from 9 to 18 h after induction, and immunostained with an anti-BrdU antibody. The percentage of BrdU-positive cells increased between 12 and 16 h in control cells, concomitant with the synchronous entry into S phase (Fig. 3B). However, Nur77 RNAi1 cells showed reduced BrdU incorporation into nuclear DNA particularly between 12 and 16 h after induction, as compared with the control cells, indicating retardation in re-entry into S phase. To determine the effect of Nur77 gene silencing on the G1 phase progression, we investigated expression of cyclins D and E. Along with cell cycle progression, cyclin D1 (a marker of mid G1), cyclin E (late G1), cyclin A (late G1/S) and cyclin B (G2/M) are sequentially activated in 3T3-L1 cells upon induction (*19*). As shown in Fig. 3C, cyclin E2 mRNA was induced 9 h after induction of differentiation, rapidly increased up to 12 h, and declined quickly to the basal level at 21 h in the control cells. However, expression of cyclin E2 in Nur77 RNAi1 cells was still at the basal level at 9 h and lower than that of control cells at 12 h. On the other hand, the expression level of cyclin E2 in the knockdown cells were higher than that of control cells at 18 and 21 h. Retarded induction of cyclin E2 in Nur77 RNAi1 cells was also confirmed by real-time RT-PCR (Fig. 3D). Similarly, induction of cyclin E1 was delayed in Nur77 RNAi1 cells (Fig. 3C). On the other hand, induction of cyclins D1 and D2 was retarded by Nur77 knockdown in early time points (Figs. 3C and 3D). In addition, the peak expression level of cyclin D1 was lower in Nur77 RNAi1 cells (Fig. 3D). Interestingly, the expression of cyclin D3 that is implicated in a cell cycle-independent role during adipogenesis (*20*) was constitutively expressed during the experimental period, and not affected by Nur77 gene silencing. These results indicate that Nur77 is involved in cell cycle progression during mitotic clonal expansion.

Ectopic Expression of Nur77 Results in Impaired Adipogenesis in 3T3-L1 Cells—Because adipocyte differentiation of 3T3-L1 cells was retarded by Nur77 knockdown, we next determined the effect of overexpression of Nur77 in 3T3-L1 cells. 3T3-L1 cells were infected with a lentiviral vector harbouring Nur77 cDNA under the control of human EF-1α gene promoter. Cells were expanded and induced to differentiate. Western blotting for 2 days postconfluent cells showed constitutive expression of Nur77 (Fig. 4A). Unexpectedly, Oil Red-O staining of the cells on day 5 demonstrated that Nur77-overexpressing cells accumulated significantly less lipid than the control cells (Fig. 4B). Consistent with lowered triacylglycerol accumulation, mRNA levels of PPARγ2, C/EBPα, adipsin, aP2 and perilipin were declined in the Nur77-overexpressing cells (Figs. 4C and 4D).

Because the knockdown experiment suggested that Nur77 regulates cell cycle progression, we speculated that these effects of overexpression of Nur77 may be due to prolonged mitotic clonal expansion. Accordingly, we next evaluated the effect of Nur77 overexpression on the cell cycle. On day 4, most of the vector control cells ceased to incorporate BrdU and began to acquire round morphology typical for adipogenic conversion, also accumulating lipid (Fig. 4E). However, a considerable number of Nur77-overexpressing cells incorporated BrdU and did not accumulate lipid on the same day, indicating that Nur77-overexpressing cells failed to exit cell cycle.

The effect of overexpression of Nur77 on the expression of cyclins D and E was determined by RT-PCR. As shown in Fig. 4F, expression of cyclin D1 was prolonged until day 5 in Nur77-overexpressing cells, in contrast to the significant decrease in the expression of this cyclin in control cells during the period of experiment. Real-time PCR analysis confirmed the enhanced expression of cyclin D1 in Nur77-overexpressing cells (Fig. 4G). Expression of cyclins E1 and E2 was less significantly different between these cells than cyclin D1, though the expression was higher in the later time points in Nur77-overexpressing cells than control cells. Cyclin D2 expression was not apparently affected by overexpression of Nur77. These results indicate that cell cycle progression during mitotic clonal expansion was prolonged by overexpression of Nur77, hereby leading to repression of differentiation.

Constitutive Expression of Nur77 Inhibits but Transient Induction of Nur77 Promotes Adipogenesis of NIH-3T3 Cells—It is known that NIH-3T3 cells have very little adipogenic potential. These cells do not differentiate when exposed to the same inducers that stimulate the differentiation of 3T3-L1 cells, but by the addition of ligands for PPARγ, considerable number of cells differentiate into adipocytes. Consistent with its lower adipogenic potential, NIH-3T3 cells exhibit lower proliferating potential in response to adipogenic induction than 3T3-L1 cells. In addition, we found that expression of Nur77 in response to differentiation induction was lower and more transient in NIH-3T3 cells than that in 3T3-L1 (data not shown). On these backgrounds, we examined the effect of constitutive expression of Nur77 on adipocyte differentiation in NIH-3T3 cells. NIH-3T3 cells were infected with a lentiviral vector harbouring Nur77 under the control of EF-1α promoter. Cells were expanded and induced for differentiation with standard hormonal cocktail together with BRL49653. Western blotting for the cells 2 days post-confluence exhibited constitutive expression of Nur77 (Fig. 5A). As shown in Fig. 5B, the above treatment induced lipid accumulation in several percents of control NIH-3T3 cells. However, Nur77-overexpressing cells were completely blocked for lipid accumulation. The overexpressing cells escaped contact inhibition, and grew piling up like transformed cells.

We next examined whether transient induction of Nur77 can promote adipocyte differentiation in NIH-3T3 cells. For this purpose, we constructed a lentiviral vector harbouring Nur77 under the control of human metallothionein IIA promoter. NIH-3T3 cells were infected with this virus, selected for zeocin resistance (a selection marker embedded in the vector) for 1 week, and expanded. Cells were then induced to differentiate with the standard hormonal cocktail together with BRL49653. In the first 6 h of differentiation induction, cells were treated with $CdCl_2$ to induce Nur77 expression. Western blotting by an enhanced detection

Downloaded from http://jb.oxfordjournals.org/ at Universidade Federal do Pará on September 29, 2012

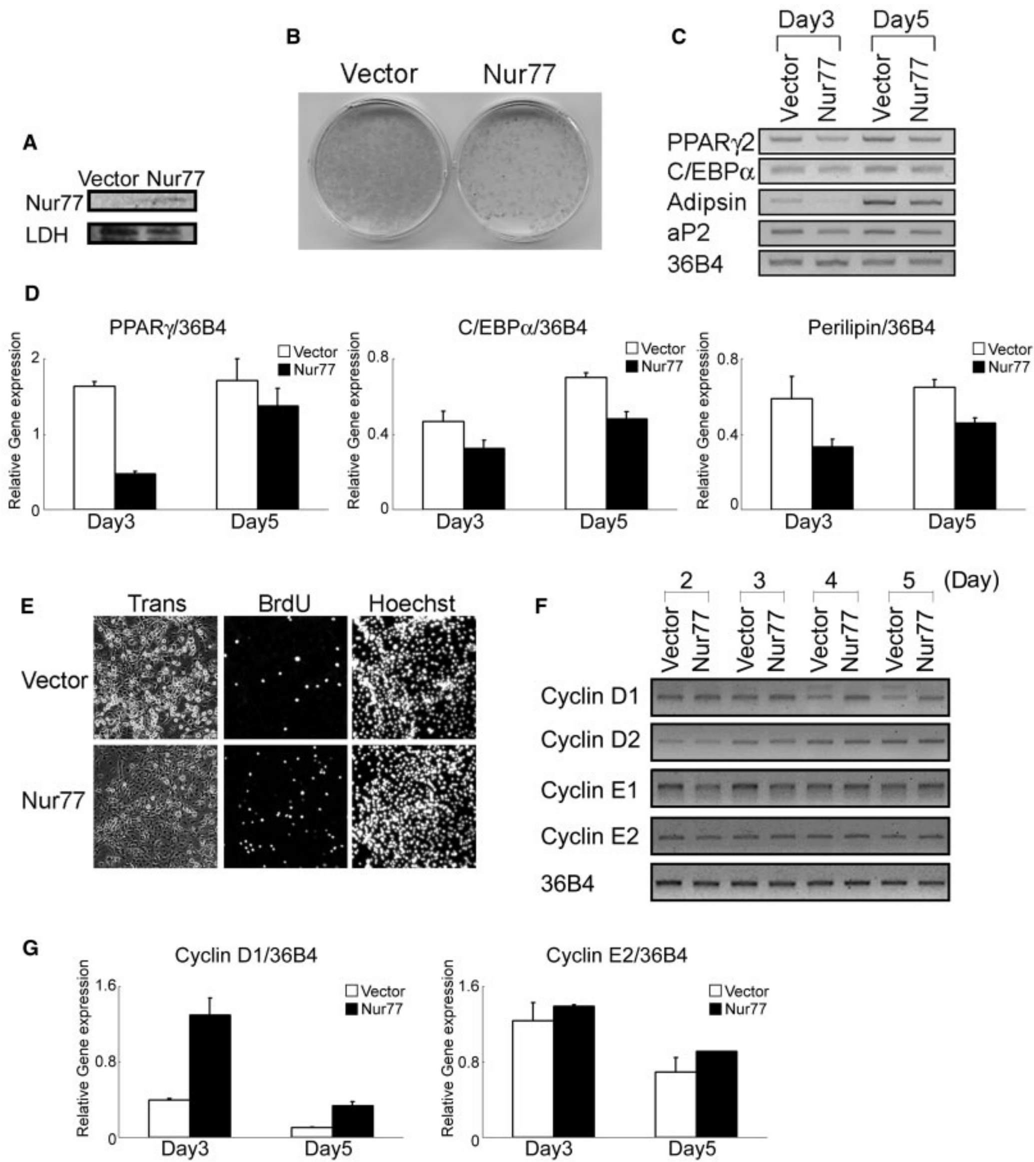

Fig. 4. **Effect of constitutive overexpression of Nur77 on the adipocyte differentiation of 3T3-L1 cells.** (A) Lentivirus-mediated overexpression of Nur77. 3T3-L1 cells were infected with an empty lentiviral vector or a vector harbouring the Nur77 cDNA under the control of human EF-1α gene promoter. Whole cell extracts prepared 2 days after confluence were analysed by immunoblotting with an anti-Nur77 antibody. LDH was used as a loading control. (B) Effect of overexpression of Nur77 on the development of lipid droplets. Nur77-overexpressing and vector control cells were induced to differentiate for 2 days. On day 5 after induction of differentiation, cells were stained with Oil Red-O. (C, D) Effect of overexpression of Nur77 on the expression of adipogenic marker genes. Total RNA was prepared from the Nur77-overexpressing and vector control cells on days 3 and 5 after induction, and subjected to RT-PCR (C) or real-time RT-PCR (D). 36B4 was used as an internal control. (E) Effect of overexpression of Nur77 on cell cycle arrest after mitotic clonal expansion. Nur77-overexpressing and vector control cells were pulsed with BrdU for 30 min on day 4 after induction of differentiation. BrdU incorporated and nuclei were visualized using an anti-BrdU antibody and Hoechst 33342, respectively. (F, G) Effect of overexpression of Nur77 on the expression levels of cyclins D and E. Relative expression levels of cyclins in Nur77-overexpressing or vector control cells on days 2–5 after induction were measured by RT-PCR (F) and real-time PCR (G). 36B4 was used as an internal control.

protocol showed induction of Nur77 for the cells carrying Cd^{2+}-inducible Nur77 gene (Fig. 5C). As shown in Fig. 5D, transient induction of Nur77 increased the number of cells accumulating lipid. That is, without Cd^{2+} treatment, a few rounded and lipid-laden cells made clusters, whereas upon Cd^{2+} treatment, the number of cells contained in these clusters increased, without no general increase in the cell number in other areas of dishes. Interestingly, there was no significant difference in the size and number of lipid droplets in the differentiated cells between the Cd^{2+}-treated and untreated cultures. These results suggest that transient induction of Nur77 promotes proliferation of the cells that are susceptible to differentiation into adipocytes, rather than enhancing adipogenic conversion of the individual cells.

Downloaded from http://jb.oxfordjournals.org/ at Universidade Federal do Pará on September 29, 2012

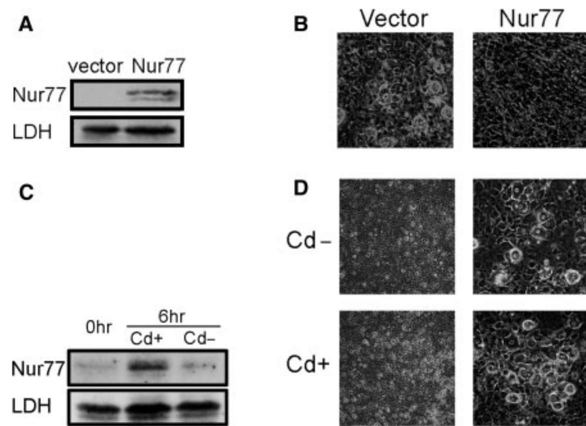

Fig. 5. **Effect of constitutive and transient expression of Nur77 on adipocyte differentiation of NIH-3T3 cells.** (A) Lentivirus-mediated constitutive overexpression of Nur77. NIH3T3 cells were infected with an empty lentiviral vector or a vector harbouring Nur77 under the control by human EF-1α gene promoter and expanded. Whole cell extracts prepared 2 days after confluence were analysed by immunoblotting with an anti-Nur77 antibody. LDH was used as a loading control. (B) Effect of constitutive overexpression of Nur77. Nur77-overexpressing and vector control cells were induced for differentiation for 2 days with the standard hormonal cocktail supplemented with BRL49653, and cultured in the differentiation medium containing BRL49653 until day 10. Adipocyte differentiation was monitored by phase-contrast microscopy. (C) Lentivirus-mediated transient expression of Nur77. NIH-3T3 cells were infected with a lentiviral vector harbouring the Nur77 cDNA under the control of human metallothionein IIA promoter, selected for zeocin resistance for 1 week, and expanded. Cells were induced to differentiate by treating with the standard hormonal cocktail, together with BRL49653 for 2 days. During the first 6 h of the treatment, cells were treated or not with $CdCl_2$. Whole cell extracts prepared before and 6 h after induction in the presence or absence of $CdCl_2$ were analyzed by immunoblotting with anti-Nur77 antibody. LDH was used as a loading control. (D) Effect of transient expression of Nur77. Cells induced to differentiate with or without $CdCl_2$ were cultured until day 10 in the presence of BRL49653. Adipocyte differentiation was monitored by phase-contrast microscopy. Left and right panels, lower and higher magnifications, respectively.

DISCUSSION

PPARγ and C/EBP family of transcription factors are known to be the master regulators of adipocyte differentiation. Among them, C/EEPβ and C/EEPδ are thought to participate in early steps of this process. However, both C/EEPβ and C/EEPδ acquire its DNA binding activity only after re-entry into S phase in mitotic clonal expansion of 3T3-L1 cells (*14*). Hence, the molecular events in the initial phase of adipocyte differentiation are still largely unclear. In the present study, we have shown that Nur77 plays a positive regulatory role in the very early phase of adipocyte differentiation.

Recent structural analysis showed that the putative ligand-binding cavity of Nur77 is occupied by bulky side chains, suggesting that Nur77 does not require an endogenous ligand (*21*). Therefore, the regulation of Nur77 expression is thought to be crucial for the control of its function. Consistent with this, expression of Nur77 is tightly regulated in 3T3-L1 cells. We demonstrated here that Nur77 mRNA was induced rapidly and transiently in the initial phase of differentiation of 3T3-L1 cells. This induction was largely dependent on FBS, and Dex slightly repressed while IBMX significantly, whereas insulin slightly, enhanced it. Nur77 was originally identified as an early gene induced by the serum in mouse fibroblasts (*16*). It was reported that Dex reduced Nur77 promoter activity in T cells (*22*) and that cAMP/PKA pathway activated the induction of Nur77 mRNA in many types of cells (*23–25*). Most importantly, we demonstrated here that Nur77 protein was expressed very transiently only in the early phase of 3T3-L1 differentiation, and this expression was completely dependent on IBMX. We presented here that Nur77 protein is rapidly degraded by proteasome during the induction of its expression in 3T3-L1 cells. IBMX seemed to protect the protein from degradation at least partially. Unfortunately, due to the extremely low level of Nur77 protein in the absence of IBMX, we could not examine the possibility that intracellular distribution and/or post-translational modification of Nur77 protein is changed by IBMX, which in turn affecting the turnover of protein.

In addition to its role as a transcription factor, Nur77 was reported to exert a proapoptoic effect in mitochondria. Therefore, regulation of the subcellular localization of Nur77 is also a crucial determinant for its function. We demonstrated here that Nur77 was localized almost exclusively in the nuclei with a punctate pattern. In contrast, C/EEPβ at this stage was localized diffusely in the nuclei in most cells and colocalized with centromeric heterochromatin in a few percent of cells, which was totally different from the distribution of Nur77. These results suggest that Nur77 plays a role in the very early stage of adipogenesis as a transcription factor, when C/EBPβ is still inactive.

We demonstrated here that inhibition of Nur77 gene expression resulted in the retardation of induction of cyclin D1 and that constitutive overexpression of Nur77 resulted in up-regulation of cyclin D1. These results strongly suggest that Nur77 regulates cyclin D1 expression, although the mechanism is not clear. On the other hand, knockdown of Nur77 resulted in delayed induction of cyclins E1 and E2, and overexpression of Nur77 resulted in slight up-regulation of these cyclins at later time points. These effects could be the secondary effect of deregulation in the processes prior to their induction, because mammalian cells exhibit sequential activation of cyclins D and E. However, liver receptor homolog (LRH)-1, another orphan nuclear receptor, was shown to induce cell proliferation through the concomitant induction of cyclins D1 and E1 (*26*). Meanwhile, though the knockdown cells of Nur77 showed retardation in the induction of cyclin D2, Nur77-overexpressing cells did not exhibit a detectable effect on cyclin D2 expression. Further analyses are required whether Nur77 regulates directly cyclins D2, E1 and E2 expression in 3T3-L1 cells.

Although early observations showed that expression of Nur77 is induced by many growth factors (*16, 27*), it remained to be determined for a long time whether

Downloaded from http://jb.oxfordjournals.org/ at Universidade Federal do Pará on September 29, 2012

Nur77 itself promotes cell proliferation. A recent study showed that Nur77 was induced by epidermal growth factor and serum, and exerted mitogenic effect in lung cancer cells (*28*). However, though the link between Nur77 and some cell cycle regulator was reported (*29–31*), the molecular mechanism by which Nur77 regulates cell proliferation is still unknown. In the present study, we provided evidence that Nur77 promotes cell cycle progression through enhancing expression of cyclins, particularly cyclin D1. Interestingly, many reports suggested that Nur77 also participates in cell cycle arrest in the G1 phase (*32–35*). In contrast, we demonstrated here that Nur77 promotes cell cycle progression in the G1 phase during clonal expansion of 3T3-L1 cells. These apparently opposite effects of Nur77 on the cell cycle progression at G1 phase raise the possibility that Nur77 is an important determinant to progress or arrest cell cycle, responding to extracellular stimuli or intracellular milieu.

In 3T3-L1 cells, mitotic clonal expansion seems to be a prerequisite for adipocyte differentiation (*18, 36*). The present results suggest that Nur77 positively regulates adipocyte differentiation by promoting mitotic clonal expansion and this effect is mediated at least in part by regulation of cyclin D1 expression. On the other hand, it has been shown that cyclin D1 itself inhibits transactivation function of PPARγ and hence adipogenesis (*37, 38*). This inhibitory effect would partly explain why prolonged expression of Nur77 represses adipocyte differentiation of 3T3-L1 and NIH-3T3 cells. Continuing proliferation is with no doubt another reason for the repressive effect on adipocyte differentiation. Hence, Nur77 promotes differentiation of 3T3-L1 cells through enhancing clonal expansion in the very early stage, while its action must be shut down rapidly for adipogenic conversion in the following stages.

There is increasing evidence that Nur77 regulates energy homeostasis in peripheral tissues. It was reported that Nur77 regulates lipolysis in skeletal muscle cells (*39*), and hepatic glucose metabolism (*40*). We added here the evidence that Nur77 promotes adipogenesis. Krüppel-like factor (KLF)-5, a transcription factor, was recently demonstrated to be essential for adipogenesis in both *in vivo* and *in vitro* (*6*). KLF5 is expressed in the early phase of adipocyte differentiation of 3T3-L1, but less transiently than Nur77. Interestingly, KLF5 was also shown to promote proliferation of NIH-3T3 cells (*41*) and activate expression of cyclin D1 (*42*). Although we observed that the mitogenic effect of Nur77 is dependent on insulin (data not shown), KLF5 exhibits the proliferative effect even in the medium only with 10% FBS (*41*). Thus, it is possible that Nur77 regulates adipogenesis in response to extracellular stimuli. It was recently reported that expression of Nur77 mRNA is relatively low, but dynamically fluctuates with a unique rhythmic pattern in white adipose tissue (*43, 44*). Although a defect has not been reported in the white adipose tissue of Nur77 knockout mice, further studies, also involving other members of the Nur family (Nurr1 and Nor-1), are required to elucidate the *in vivo* roles of Nur77 in adipocyte differentiation.

We thank Drs H. Miyoshi, S. Taketani and N. Usuda for providing plasmids or an antibody. This work was supported in part by a Grant-in Aid for Scientific Research from the Japan Society for the Promotion of Science and the 21st Century COE Program.

REFERENCES

1. Flier, J.S. (2004) Obesity wars: molecular progress confronts an expanding epidemic. *Cell* **116**, 337–350
2. Rosen, E.D. and Spiegelman, B.M. (2000) Molecular regulation of adipogenesis. *Annu. Rev. Cell Dev. Biol.* **16**, 145–171
3. Rosen, E.D., Sarraf, P., Troy, A.E. *et al.* (1999) PPAR gamma is required for the differentiation of adipose tissue in vivo and in vitro. *Mol. Cell* **4**, 611–617
4. Wang, N.D., Finegold, M.J., Bradley, A. *et al.* (1995) Impaired energy homeostasis in C/EBPα knockout mice. *Science* **269**, 1108–1112
5. Tanaka, T., Yoshida, N., Kishimoto, T. *et al.* (1997) Defective adipocyte differentiation in mice lacking the C/EBPβ and/or C/EBPδ gene. *EMBO J.* **16**, 7432–7443
6. Oishi, Y., Manabe, I., Tobe, K. *et al.* (2005) Krüppel-like transcription factor KLF5 is a key regulator of adipocyte differentiation. *Cell Metab.* **1**, 27–39
7. Chen, Z., Torrens, J.I., Anand, A. *et al.* (2005) Krox20 stimulates adipogenesis via C/EBPβ-dependent and -independent mechanisms. *Cell Metab.* **1**, 93–106
8. Hsu, H.C., Zhou, T., and Mountz, J.D. (2004) Nur77 family of nuclear hormone receptors. *Curr. Drug Targets Inflamm. Allergy* **3**, 413–423
9. Inuzuka, H., Nanbu-Wakao, R., Masuho, Y. *et al.* (1999) Differential regulation of immediate early gene expression in preadipocyte cells through multiple signaling pathways. *Biochem. Biophys. Res. Commun.* **265**, 664–668
10. Soukas, A., Socci, N.D., Saatkamp, B.D. *et al.* (2001) Distinct transcriptional profiles of adipogenesis in vivo and in vitro. *J. Biol. Chem.* **276**, 34167–34174
11. Fu, M., Sun, T., Bookout, A.L. *et al.* (2005) A Nuclear Receptor Atlas: 3T3-L1 adipogenesis. *Mol. Endocrinol.* **19**, 2437–2450
12. Hashiguchi, N., Kojidani, T., Imanaka, T. *et al.* (2002) Peroxisomes are formed from complex membrane structures in PEX6-deficient CHO cells upon genetic complementation. *Mol. Biol. Cell* **13**, 711–722
13. Li, H., Kolluri, S.K., Gu, J. *et al.* (2000) Cytochrome c release and apoptosis induced by mitochondrial targeting of nuclear orphan receptor TR3. *Science* **289**, 1159–1164
14. Tang, Q.Q. and Lane, M.D. (1999) Activation and centromeric localization of CCAAT/enhancer-binding proteins during the mitotic clonal expansion of adipocyte differentiation. *Genes. Dev.* **13**, 2231–2241
15. Hazel, T.G., Nathans, D., and Lau, L.F. (1988) A gene inducible by serum growth factors encodes a member of the steroid and thyroid hormone receptor superfamily. *Proc. Natl. Acad. Sci. USA* **85**, 8444–8448
16. Lau, L.F. and Nathans, D. (1985) Identification of a set of genes expressed during the G0/G1 transition of cultured mouse cells. *EMBO J.* **4**, 3145–3151
17. Qiu, Z., Wei, Y., Chen, N. *et al.* (2001) DNA synthesis and mitotic clonal expansion is not a required step for 3T3-L1 preadipocyte differentiation into adipocytes. *J. Biol. Chem.* **276**, 11988–11995
18. Tang, Q.Q., Otto, T.C., and Lane, M.D. (2003) Mitotic clonal expansion: a synchronous process required for adipogenesis. *Proc. Natl. Acad. Sci. USA* **100**, 44–49
19. Morrison, R.F. and Farmer, S.R. (1999) Role of PPARγ in regulating a cascade expression of cyclin-dependent kinase inhibitors, p18(INK4c) and p21(Waf1/Cip1), during adipogenesis. *J. Biol. Chem* **274**, 17088–17097

Downloaded from http://jb.oxfordjournals.org/ at Universidade Federal do Pará on September 29, 2012

20. Sarruf, D.A., Iankova, I., Abella, A. *et al.* (2005) Cyclin D3 promotes adipogenesis through activation of peroxisome proliferator-activated receptor gamma. *Mol. Cell. Biol.* **25**, 9985–9995
21. Flaig, R., Greschik, H., Peluso-Iltis, C. *et al.* (2005) Structural basis for the cell-specific activities of the NGFI-B and the Nurr1 ligand-binding domain. *J. Biol. Chem.* **280**, 19250–19258
22. Woronicz, J.D., Lina, A., Calnan, B.J. *et al.* (1995) Regulation of the Nur77 orphan steroid receptor in activation-induced apoptosis. *Mol. Cell Biol.* **15**, 6364–6376
23. Lim, R.W., Yang, W.L., and Yu, H. (1995) Signal-transduction-pathway-specific desensitization of expression of orphan nuclear receptor TIS1. *Biochem. J.* **308**, 785–789
24. Kovalovsky, D., Refojo, D., Liberman, A.C. *et al.* (2002) Activation and induction of NUR77/NURR1 in corticotrophs by CRH/cAMP: involvement of calcium, protein kinase A, and MAPK pathways. *Mol. Endocrinol.* **16**, 1638–1651
25. Fass, D.M., Butler, J.E., and Goodman, R.H. (2003) Deacetylase activity is required for cAMP activation of a subset of CREB target genes. *J. Biol. Chem.* **278**, 43014–43019
26. Botrugno, O.A., Fayard, E., Annicotte, J.S. *et al.* (2004) Synergy between LRH-1 and beta-catenin induces G1 cyclin-mediated cell proliferation. *Mol. Cell* **15**, 499–509
27. Ryseck, R.P., Macdonald-Bravo, H., Mattei, M.G. *et al.* (1989) Structure, mapping and expression of a growth factor inducible gene encoding a putative nuclear hormonal binding receptor. *EMBO J.* **8**, 3327–3335
28. Kolluri, S.K., Bruey-Sedano, N., Cao, X. *et al.* (2003) Mitogenic effect of orphan receptor TR3 and its regulation by MEKK1 in lung cancer cells. *Mol. Cell Biol.* **23**, 8651–8667
29. Chan, F.K., Zhang, J., Cheng, L. *et al.* (1995) Identification of human and mouse p19, a novel CDK4 and CDK6 inhibitor with homology to p16ink4. *Mol. Cell Biol.* **15**, 2682–2688
30. Mu, X. and Chang, C. (2003) TR3 orphan nuclear receptor mediates apoptosis through up-regulating E2F1 in human prostate cancer LNCaP cells. *J. Biol. Chem.* **278**, 42840–42845
31. Pei, L., Castrillo, A., and Tontonoz, P. (2006) Regulation of macrophage inflammatory gene expression by the orphan nuclear receptor Nur77. *Mol. Endocrinol.* **20**, 786–794
32. Castro, D.S., Hermanson, E., Joseph, B. *et al.* (2001) Induction of cell cycle arrest and morphological differentiation by Nurr1 and retinoids in dopamine MN9D cells. *J. Biol. Chem.* **276**, 43277–43284
33. Wu, Q., Liu, S., Ye, X.F. *et al.* (2002) Dual roles of Nur77 in selective regulation of apoptosis and cell cycle by TPA and ATRA in gastric cancer cells. *Carcinogenesis* **23**, 1583–1592
34. Arkenbout, E.K., de Waard, V., van Bragt, M. *et al.* (2002) Protective function of transcription factor TR3 orphan receptor in atherogenesis: decreased lesion formation in carotid artery ligation model in TR3 transgenic mice. *Circulation* **106**, 1530–1535
35. Arkenbout, E.K., van Bragt, M., Eldering, E. *et al.* (2003) TR3 orphan receptor is expressed in vascular endothelial cells and mediates cell cycle arrest. *Arterioscler. Thromb. Vasc. Biol.* **23**, 1535–1540
36. Patel, Y.M. and Lane, M.D. (2000) Mitotic clonal expansion during preadipocyte differentiation: calpain-mediated turnover of p27. *J. Biol. Chem.* **275**, 17653–17660
37. Wang, C., Pattabiraman, N., Zhou, J.N. *et al.* (2003) Cyclin D1 repression of peroxisome proliferator-activated receptor gamma expression and transactivation. *Mol. Cell Biol.* **23**, 6159–6173
38. Fu, M., Rao, M., Bouras, T. *et al.* (2005) Cyclin D1 inhibits peroxisome proliferator-activated receptor gamma-mediated adipogenesis through histone deacetylase recruitment. *J. Biol. Chem.* **280**, 16934–16941
39. Maxwell, M.A., Cleasby, M.E., Harding, A. *et al.* (2005) Nur77 regulates lipolysis in skeletal muscle cells. Evidence for cross-talk between the beta-adrenergic and an orphan nuclear hormone receptor pathway. *J. Biol. Chem.* **280**, 12573–12584
40. Pei, L., Waki, H., Vaitheesvaran, B. *et al.* (2006) NR4A orphan nuclear receptors are transcriptional regulators of hepatic glucose metabolism. *Nat. Med.* **12**, 1048–1055
41. Sun, R., Chen, X., and Yang, V.W. (2001) Intestinal-enriched Krüppel-like factor (Krüppel-like factor 5) is a positive regulator of cellular proliferation. *J. Biol. Chem.* **276**, 6897–6900
42. Nandan, M.O., Yoon, H.S., Zhao, W. *et al.* (2004) Krüppel-like factor 5 mediates the transforming activity of oncogenic H-Ras. *Oncogene* **23**, 3404–13
43. Bookout, A.L., Jeong, Y., Downes, M. *et al.* (2006) Anatomical profiling of nuclear receptor expression reveals a hierarchical transcriptional network. *Cell* **126**, 789–799
44. Yang, X., Downes, M., Yu, R.T. *et al.* (2006) Nuclear receptor expression links the circadian clock to metabolism. *Cell* **126**, 801–810

Downloaded from http://jb.oxfordjournals.org/ at Universidade Federal do Pará on September 29, 2012